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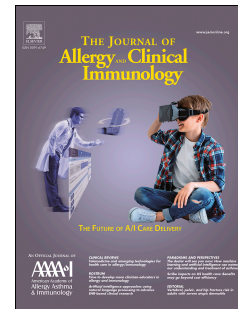
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Unraveling heterogeneity in pediatric atopic dermatitis: identification of serum biomarker based patient clusters

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Conflicts of interest

D. Bakker, E. Knol, and J. Thijs have been speakers for Sanofi Genzyme. M. de Graaf has been principal investigator and advisory board member for Sanofi Genzyme and Regeneron Pharmaceuticals, Inc; and has been advisory board member for Eli Lilly. M. De Bruin-Weller has been a consultant, advisory board member, and/or speaker for AbbVie, Almirall, Eli Lilly, Galderma, Janssen, Leo Pharma, Pfizer, Regeneron, Sanofi-Genzyme, and UCB. All other authors declare no relevant conflicts of interest.

ABSTRACT

Background: Increasing evidence shows that pediatric AD differs from adult AD on a biological level. Broad biomarker profiling across a wide range of ages of pediatric AD patients is lacking.

Objective: To identify serum biomarker profiles in AD children aged 0–17 years and compare these with previously found profiles in adult AD.

Methods: Luminex multiplex immunoassays were used to measure 145 biomarkers in serum from 240 AD children (aged 0–17 years). Principal components analysis followed by unsupervised k-means clustering were performed to identify patient clusters. Patients were stratified into age groups (0-4 years, 5-11 years, and 12-17 years) to assess association between age and cluster membership.

Results: Children aged 0-4 years had highest levels of Th1-skewing markers, and lowest levels of Th17-related markers. Th2-related markers did not significantly differ between age groups. Similarly to adults, cluster analysis identified four distinct pediatric patient clusters (“Th2/retinol dominant”, “skin-homing dominant”, “Th1/Th2/Th17/IL-1 dominant”, and “Th1/IL-1/eosinophil inferior” cluster). Only the “Th1/Th2/Th17/IL-1 dominant” cluster resembled one of the previously identified adult clusters. Although no association with age or age of onset seemed to be found, disease severity was significantly associated with the "skin-homing dominant" cluster.

Conclusion: Four distinct patient clusters based on serum biomarker profiles could be identified in a large pediatric AD cohort, of which one was similar to previously identified adult clusters. The identification of endotypes driven by distinct underlying immunopathological pathways might be useful to define pediatric AD patients at risk of persistent disease and may necessitate different targeted treatment approaches.

Key messages

- In a large cohort of AD patients aged 0 – 17 years, we identified four patient clusters based on distinct serum biomarker profiles.
- Pediatric AD clusters were influenced by disease severity, and not by age or age of onset. Only one pediatric AD cluster was comparable to one of the previously identified adult AD clusters.
- Stratification of pediatric AD patients into distinct biomarker-based endotypes might help to predict persistent disease, and may contribute to more personalized treatment approaches.

Capsule summary

By identifying four pediatric AD patient clusters based on distinct serum biomarker profiles, and comparing them with adult clusters, our study provides insight in the immuno-pathological characterization of pediatric AD, which might contribute to more personalized medicine in future.

Key words: Atopic dermatitis; pediatric; biomarkers; endotypes; personalized medicine; principal components analysis; cluster analysis.

ABBREVIATIONS

37	AD	Atopic dermatitis
38	ANOVA	ANalysis Of Variance
39	CCL	C-C Motif chemokine ligand
40	CTACK	Cutaneous T cell- attracting chemokine
41	CXCL	C-X-C motif chemokine ligand
42	EASI	Eczema Area Severity Index
43	FDR	False Discovery Rate
44	TACI	Transmembrane activator calcium modulator and cyclophilin ligand interactor
45	TARC	Thymus and activation regulated chemokine
46	Th	T-helper
47	TNF	Tumor Necrosis Factor
48	TWEAK	tumor necrosis factor-like weak inducer of apoptosis
49	IFN	Interferon
50	IL	Interleukin
51	MCP-1	Monocyte Chemoattractant Protein-1
52	MDC	Macrophage-derived chemokine
53	MMP	Matrix metalloproteinase
54	MIF	Migration Inhibitory Factor
55	PARC	Pulmonary and activation-regulated chemokine
56	PCA	Principal Components Analysis
57	RBP4	Retinol binding protein 4
58	SD	Standard Deviation
59	sICAM	soluble Intercellular Adhesion Molecule
60	WSS	Within-cluster sum of square

61 INTRODUCTION

62 Atopic dermatitis (AD) is the most common chronic inflammatory skin disease, affecting up to 20% of

children and up to 10% of adults.¹⁻³ AD can present at all ages, but mostly begins in early childhood. Although the general consensus is that most pediatric AD patients will eventually ‘outgrow’ the disease, recent studies suggest that persistence into adulthood is more common than previously recognized.⁴ The clinical presentation and distribution of AD in childhood and adults is clearly different⁵⁻⁷, and atopic comorbidities, including food allergy, asthma, and allergic rhinitis, develop over the course of infancy and childhood, described as the ‘atopic march’.^{8,9} Besides the well-known differences in clinical presentation, increasing insights into blood and skin profiles show substantial differences between pediatric and adult AD.¹⁰⁻¹³ Although both populations show significant Th2 activation in skin and blood, early-onset pediatric AD also shows Th17/Th22 skewing, but lacks the Th1 upregulation that is seen in adults.^{10, 12, 13}

In the past decade it has become increasingly clear that adult AD is not only heterogeneous based on clinical characteristics, but that different pathophysiological mechanisms can be defined in different subgroups of patients. In recent studies, we identified four clearly differentiated adult AD patient clusters, each characterized by a unique serum biomarker profile.^{14, 15} These clusters might represent endotypes in which the disease is driven by a distinct underlying mechanism. However, heterogeneity on a biological level has not yet been confirmed in pediatric AD. While most pediatric biomarker data are based on studies in infants and young children with recent-onset AD, broad blood profiling in all age ranges of pediatric AD patients is limited.¹⁰⁻¹³

The increasing understanding of molecular pathways involved in chronic AD has accelerated the development of more targeted systemic therapies for adult and adolescent AD patients, and will eventually move to children.¹⁶ The specific biomarker pathways distinguishing different patient clusters may be particularly meaningful for the application of molecularly targeted drugs, and defining the most optimal treatment for the individual patient, since different endotypes might respond differently to the particular treatments. In adult AD, few single biomarkers have previously been proposed for the prediction of response to targeted therapies.¹⁷⁻¹⁹ However, no data have yet been published for pediatric

AD. Besides predicting treatment response, biomarker profiling could also be used for different purposes in children, including early diagnosing of AD, the identification of patients with high risk of persistent disease, and prediction of side effects for a given drug.²⁰ This necessitates even more the identification of pediatric AD endotypes to optimize safe and effective personalized treatment approaches. Early treatment and AD control in pediatric patients may impact the natural history of the disease. In the present study, we investigated biomarker profiles in children with AD aged between 0 and 17 years and compared these profiles with the previously found adult AD endotypes. We expect that by defining biomarker profiles in pediatric AD, we will eventually be able to predict the course of the disease and optimize personalized medicine approaches.

METHODS

Study design and data collection

Patients and samples

Serum samples from 240 children aged 0 – 17 years, diagnosed with AD as defined by the criteria of Hanifin and Rajika²¹, were retrospectively selected. Sera were collected at the Wilhelmina Children's Hospital (University Medical Center Utrecht) between 2014 – 2017 and stored at -80 degrees Celsius in a biobank until analysis. Exclusion criteria for this study were use of systemic immunosuppressive drugs within four weeks before blood sampling. Disease severity was assessed by the Eczema Area and Severity Index (EASI) score. Clinical characteristics were retrospectively extracted from the patients' electronic medical records. Before study inclusion, parents signed institutional review board-approved written consent, according to the Declaration of Helsinki principles. Protocols of this study were approved by the Institutional Review Board of the University Medical Center Utrecht (Utrecht, The Netherlands).

Serum biomarkers

To characterize disease heterogeneity and to identify specific pediatric AD patient clusters, we quantified 145 analytes by a multiplex immunoassay based on Luminex technology²² as previously described¹⁵,

using an in-house validated panel of analytes listed in supplementary Table E1. The panel was selected based on our previous studies in adult AD with the addition of two markers newly available.^{14, 15} Serum samples that were above or below the assay limits of detection were given values equivalent to the lower limit of quantification divided by two or the upper limit of quantification multiplied by two.

Statistical analyses

Serum biomarker data was cleansed of patients with any missing data (1 patient was removed) and subjected to Box-Cox transformation before analyses. To identify differences in serum biomarker levels within different age groups, patients were stratified into three groups: 0 – 4 years, 5 – 11 years, and 12 – 17 years.

The normalized biomarker data were analyzed using a principal components analysis (PCA) to reduce the dimensionality of the data and find the optimal number of principal components explaining the majority of variance in the data, followed by k-means cluster analysis to define and visualize clusters in those principal components, as previously described.^{14, 15} The optimal number of clusters was determined by using the elbow method, which looks at the total within-cluster sum of square (WSS) as a function of the number of clusters.²³ The optimal number of clusters was selected to be such that adding another cluster does not significantly reduce the total WSS. To investigate the differences between the age groups, we looked *a posteriori* if the defined clusters were associated with the age groups. Additionally, we compared the biomarker profiles found in our pediatric AD cohort with the previously described biomarker profiles in adults AD patients.^{14, 15}

Clinical characteristics and serum biomarker levels between the age groups and patient clusters were compared using chi-square tests for categorical variables or one-way ANOVA for continuous variables, followed by pairwise t-tests or chi-square tests when appropriate. Benjamini-Hochberg correction was used for all multiple comparisons, controlling False Discovery Rate (FDR). FDR adjusted P-values <0.05

were considered statistically significant. The association of serum biomarkers with disease severity was evaluated using Pearson correlation coefficients. All statistical analyses were performed using R Project software (version 3.4.1)²⁴.

RESULTS

Patient characteristics and age groups

A total of 240 pediatric AD patients (mean age 8.2 years, SD= 5.5) were included. AD disease severity at the moment of sampling ranged from clear to severe, with a mean EASI score of 14.6 (SD=10.7). Clinical characteristics are summarized in Table 1. Disease severity was not significantly different between children aged 0 – 4 years old (n=77, mean EASI 13.3, SD=10.1), 5 – 11 years (n=84, mean EASI 14.3, SD=11.3), and 12 – 17 years (n=79, mean EASI 16.2, SD=10.5)(Table 2). The presence of other atopic comorbidities, including asthma (p=0.001), allergic rhinitis (p<0.001) and food allergy (p=0.050) was significantly higher in the oldest age group (chi-square test). The youngest age group (0 – 4 years) included significantly more males compared to the 5 – 11 years and 12 – 17 years groups (55.8%, compared to 31.0%, and 39.2%, respectively, p=0.005, chi-square test).

Serum biomarker levels were first compared between the three age groups using a one-way ANOVA, followed by pairwise t-tests with Benjamini-Hochberg correction for multiple comparisons (Figure 1 and supplementary Table E2). By applying this supervised approach, the youngest children, aged 0 – 4 years, were characterized by the highest levels of innate, mostly Th1-skewing markers (IL-18, MCP1/CCL2, TNF receptor 2), epithelial proliferation and differentiation (epidermal growth factor [EGF]), B-cell homing (BLC/CXCL13), adhesion molecules (P-selectin, sICAM), the adipokine adiponectin, and the proinflammatory cytokine macrophage migration inhibitory factor (MIF). Children aged 0 – 4 years were also characterized by the lowest levels of the Th17-related marker trappin2/elafin. Children aged 5 – 11 years were distinguished from the other age groups by the highest levels of the TNF superfamily members

TWEAK/TNFSF12 and TACI/TNFRSF13B. The oldest children (12 – 17 years old) were characterized by the highest serum levels of markers related to tissue remodeling (MMP-1, MMP-3, MMP-9), and the lowest levels of the adhesion molecule sICAM and the multifunctional glycoprotein osteopontin (OPN). Th2-related (IL-5, IL-13, TARC/CCL17, MDC/CCL22, MCP-4/CCL13) and Th22-related (IL-22) markers were higher expressed in children aged 0 – 4 years compared to the other age groups, however, not statistically significant.

Correlation of biomarkers with disease severity

We next investigated which serum biomarkers were associated with AD disease severity by determining correlation of each measured serum biomarker with EASI scores in all 240 pediatric patients (Figure 2). Significant positive correlations between disease severity were found for PARC/CCL18 ($r=0.63$), apelin ($r=0.53$), IL-1R2 ($r=0.49$), TARC/CCL17 ($r=0.48$), MMP-1 ($r=0.48$), CTACK ($r=0.41$), elastase ($r=0.40$), I309 ($r=0.40$), MDC ($r=0.38$), sVCAM ($r=0.36$), E-selectin ($r=0.36$), IL-22 ($r=0.31$), and S100A8 ($r=0.31$). EASI score was significantly negatively correlated with RBP4 ($r=-0.68$), CatS ($r=-0.66$), ACE ($r=-0.48$), IL-25 ($r=-0.36$), IL-26 ($r=-0.36$), NAP2 ($r=-0.35$), and MMP-8 ($r=-0.30$). Overall, correlation coefficients from these markers with disease severity were comparable between the three age groups and the total cohort (Supplementary Table E3).

Characterization of pediatric AD clusters

In the next step, unsupervised analyses were performed on the Box-Cox transformed serum biomarker data of all 240 pediatric AD patients to identify distinct patient clusters based on serum biomarker profiles. After PCA, the cumulative percentage of variance showed that the first 50 principal components described at least 90% of the dataset's variance (Supplemental Figure E2A), and were, hence, included in the unsupervised k-means cluster analysis (top 20 markers for the first three PC's in Supplemental Table E4). By applying the elbow method on the k-means clustering, four clusters were indicated as the

appropriate number of clusters (Supplemental Figure E2B and Figure 3A). Clinical characteristics were compared between the four clusters (Table 3). Pediatric AD patient clusters seem not to be influenced by age, as age did not significantly differ between the four clusters (Table 3, $p=0.11$, one-way ANOVA), and patients from the three age groups were equally divided over the four clusters (Figure 3B, $p=0.074$, chi-square test).

Averages of serum biomarker levels were calculated per cluster, and compared using a one-way ANOVA, followed by pairwise t-tests with Benjamini-Hochberg correction for multiple comparisons, to characterize the biomarker profiles driving the four clusters (Figure 4; supplementary Table E5). Cluster 1 was the largest cluster, representing 41% of the pediatric AD population. Cluster 1 patients had a mean age of 8.7 years (SD=5.7 years) and had the lowest mean EASI score (mean 9.2 years, SD=5.4). This cluster was distinct from the other three by having the highest levels of the acute phase protein retinol binding protein 4 (RBP4). Besides, the Th2 cytokines IL-4, IL-5, IL-13, and TSLP, the Th17-related cytokines IL-23 and IL-26 were higher in cluster 1 compared to cluster 2 and 4, but lower compared to cluster 3. Cluster 1 could be defined as the “Th2/retinol dominant” cluster.

Cluster 2 represented 31% of the patients and had a mean age of 8.8 years (SD=5.3 years). Patients in this cluster had a significantly more severe AD compared to the other clusters ($p<0.001$), with a mean EASI score of 27.8 (SD=7.5). This cluster also had the highest incidence of food allergy (53.4%). Its biomarker profile was characterized by the highest levels of apelin, and markers related to skin-homing (PARC/CCL18, TARC/CCL17, and CTACK/CCL27), and the lowest levels of markers related to tissue remodeling and angiogenesis (adiponectin, MMP-8, TIMP1). All of these markers were also strongly correlated with EASI score. Cluster 2 was defined as the “skin homing dominant” cluster.

Cluster 3 represented 18% of the patients, had a mean age of 6.9 years (SD=5.4 years) and mean EASI score of 10.5 (SD=9.1). Cluster 3 was uniquely defined by having the highest levels of biomarkers related to the Th1 pathway (IL-2, IL-12, IFN α , IFN γ , TNF α , TNF β , MIG/CXCL9, ITAC/CXCL11), the Th2

pathway (IL-4, IL-5, IL-13, eotaxin-3/CCL26, TSLP, MCP-4/CCL13), the Th17 pathway (IL-23, IL-26, MIP3a/CCL20, GM-CSF), the IL-1 family pathway (IL-1 α , IL-1R α , IL-1R1, IL-18BP α , IL-37), the TNF superfamily pathway (TNFR1, TNFR2, TWEAK/TNFSF12, LIGHT/TNFSF14), and T-cell activation (sIL2R α). Cluster 3 could be described as the “Th1/Th2/Th17/IL-1 dominant” cluster.

Cluster 4 represented 10% of the patients, mean age in this cluster was 6.6 years (SD= 4.9 years) and mean EASI score 12.3 (SD=9.1). Patients from this cluster had the lowest incidence of food allergy (24.0%). Regarding the serum biomarker profile, cluster 4 was distinct from the other three clusters by having the highest levels of the chemokines RANTES/CCL5 and PF4/CXCL4, and the monocyte activation marker soluble CD14. Besides, cluster 4 showed the lowest levels of biomarkers related to the Th1 pathway (MIG/CXCL9, ITAC/CXCL11, and MIP1b/CCL2), eosinophil trafficking (eotaxin-1/CCL11 and eotaxin-3/CCL26), the IL-1 family pathway (IL1R1, IL-18BP α), the TNF superfamily pathway (TNFR1, TNFR2, TWEAK/TNFSF12), neutrophil activation and trafficking (elastase, GCP2), and T-cell activation and skin-homing (sIL2R α , CTACK). This cluster was defined as the “Th1/IL-1/eosinophil inferior” cluster.

In summary, we could identify four distinct pediatric AD patient clusters. Two of the four clusters showed skewing towards the Th2 pathway (cluster 1 and 3), of which cluster 3 was further characterized by a strong immune activation state, related to both innate as well as T-cell immunity. Cluster 2 was clinically defined by the highest EASI score and was characterized by a biomarker profile skewed towards skin-homing-related markers. Besides elevation of few innate immunity-related markers, cluster 4 was overall distinguished by a relatively low inflammatory state.

DISCUSSION

This is the first study to broadly characterize serum biomarker profiles in a large cohort of pediatric AD patients aged 0 - 17 years. We confirmed heterogeneity at the level of serum biomarkers in pediatric AD

patients and identified four patient clusters based on their unique systemic immune profiles, by using an unsupervised clustering approach. Our results suggest unique endotypes in pediatric AD patients, possibly arguing for personalized, endotype-driven therapeutic approaches, rather than the currently used “one-size-fits-all” concept.

Blood biomarker profiles of early-onset pediatric AD have previously been characterized by an upregulation of Th2, Th17 and tissue remodeling markers, and lacked the Th1 upregulation that is seen in adult AD.^{10, 11, 13, 25} In contrast to these findings, our pediatric AD patients aged 0 – 4 years, corresponding in age to the previously studied early pediatric AD cohorts^{10-13, 26}, was characterized by higher expression of innate activation markers, mostly related to Th1, and decreased levels of the Th17 marker trappin/elafin, compared to the older children. The prior studies characterized blood profiles of pediatric AD patients within 6 months after disease onset compared to age-matched healthy controls, which might explain the different findings. Th1-related markers have been identified as marker for disease chronicity and immune development, but in the view of our findings, they might also represent other immune-related mechanisms distinguishing infant and toddler AD from older children and adolescents.²⁷ The innate activation markers significantly upregulated in the youngest group (IL-18, MCP-1/CCL2, and TNFR2) have been proven to contribute to both Th1 and Th2 cytokine-mediated inflammation. Besides, IL-18 and MCP-1/CCL2 are associated with severity of pediatric AD.²⁸⁻³² Pediatric AD is supposed to be an even more Th2-dominant disease than adult AD. Although not significant, other Th2-related markers including IL-5, IL-13, TARC/CCL17, MDC, and MCP-4 were higher expressed in the youngest children, compared to the 5 – 17 years old children. As AD is a primarily Th2-driven disease, it could be that Th2 cytokines are upregulated in all pediatric AD patients, and therefore not different within the three age groups. Serum samples from age-matched healthy controls are needed to further investigate this.

The previously described positive correlations of pediatric AD severity with TARC/CCL17, PARC/CCL18, CTACK/CCL27, MDC/CCL22, E-selectin, and the IL-1 decoy receptor IL-1R2 were also present in our study.^{10, 33-35} MMP-1, an inflammatory marker related to tissue remodeling, and previously described to be

negatively associated with skin scores in early-onset AD patients (mean age 1.8 , SD= 1.6 years)¹⁰, showed positive correlation with EASI in our cohort. In a previous study by Thijs et al.³⁶ MMP-1 also showed a significant positive correlation with disease severity in adult AD. The difference in the direction of the correlation of MMP-1 with disease severity may therefore reflect age and chronicity of the disease. Retinol binding protein (RBP)4 showed a strong negative correlation with EASI scores in our cohort. Both lower retinol levels and RBP4 expression have been detected in skin samples of adult AD, and a negative association of serum retinol with AD severity was reported in children.^{37, 38} Retinol has important immunomodulatory effects, and decreased levels of RBP and vitamin A are associated with infection and inflammation.³⁹⁻⁴¹ These data might support the negative correlation with EASI in our pediatric cohort as an effect of excessive skin inflammation. In our large pediatric AD cohort, EASI was scored by several different physicians. The subsequent higher inter-rater variability might have resulted in relatively lower correlation coefficients in our study.

In contrast to previous studies investigating blood and skin biomarkers in pediatric AD¹⁰⁻¹³, we included a large cohort of AD children across a wide range of ages and disease duration and used a quantitative method to measure a broad panel of serum biomarkers. By using an unsupervised clustering approach, we could identify four pediatric AD patient clusters, characterized by specific serum biomarkers that were significantly differentially expressed compared to the other clusters. Patients stratified in cluster 1 had the lowest disease severity and were characterized by the highest levels of RBP4, which showed the strongest negative correlation with EASI. Additionally, cluster 1 showed higher levels of IL-4, IL-5, IL-13 and TSLP, and could be defined as the “Th2/retinol dominant” cluster. Patients in cluster 1 and 3, representing 59% of the patients, shared a Th2-cytokine high profile, corresponding to the percentage of Th2-dominant patients as previously reported in adults.^{14, 15} These patients would hypothetically be the most ideal candidates for Th2 targeting drugs.

In contrast to the other three clusters, cluster 4 showed a relatively low inflammatory state, with no clear immune skewing, and could be distinguished from the other clusters as being the “Th1/IL-1/eosinophil

inferior” cluster. Cluster 4 was defined by elevation of the monocyte activation markers RANTES/CCL5, PF4/CXCL4, and sCD14. Elevated platelet activation, as shown by higher levels of PF4/CXCL4, has been suggested to play a role in the pathomechanism of chronic skin inflammation in AD, by inducing leukocyte recruitment and through direct activation of local capillary endothelial cells and attraction of effector T-cells to the skin.⁴² Both RANTES/CCL5, a potent eosinophil, monocyte, basophil and lymphocyte chemo-attractant, and the monocyte activation marker sCD14 have shown evidence of association with AD as well.⁴³⁻⁴⁵

By focusing on the driving pathways per patient cluster, only one out of the four pediatric AD clusters was comparable to one of the previously defined endotypes in adult AD patients.^{14, 15} The biomarker profile of pediatric “Th1/Th2/Th17/IL-1 dominant” cluster 2 corresponds to the profile of the “Th1/Th2/Th17 dominant” cluster identified in adult AD patients. These results strengthen the previous findings that blood profiles in pediatric AD differ from adult AD patients. However, the identified biomarker-based pediatric patient clusters in the current study were not influenced by age or age of onset. Furthermore, the absolute differences in biomarker levels between the four unsupervised identified clusters were more pronounced than the differences that were found by using a supervised approach to compare the three age groups.. Although three of the pediatric patient clusters differ from the previously identified adult AD clusters, our results might indicate that the distinct pathophysiologic mechanisms driving the heterogeneity of pediatric AD cannot be solely assigned to the difference in age or duration of the disease, and argue for endotype-specific, rather than uniform or age-specific therapeutic strategies.

One of the most important questions regarding disease heterogeneity in pediatric AD is in which patient the disease will resolve and in whom it will persist into adulthood. One could speculate that patients with resolving childhood AD and persisting disease may represent separate endotypes. Early identification and targeted treatment of the non-resolving endotype might theoretically prevent the atopic march and persistence of AD into adulthood. Previous birth cohort studies have shown that one of the strongest risk factor for non-resolving AD is disease severity, and that the presence of asthma and allergic rhinitis did not

affect the course of AD.⁴⁶⁻⁴⁸ In contrast to the previous studies in adults^{14, 15}, our current study showed that cluster membership of pediatric AD patients was influenced by disease severity. Patients in cluster 3 had significantly higher EASI scores compared to the other three clusters. Their driving biomarker profile was characterized by the highest levels of the Th2 cytokine PARC/CCL18 and apelin, and the lowest levels of RBP4, MMP-8, and ACE, all biomarkers that were significantly correlated with EASI scores. Patients in cluster 3 represented 31% of the total cohort, which is consistent with data from large birth cohorts showing that up to one third of the children diagnosed with AD had persistent disease.⁴⁶⁻⁴⁸ On the other hand, patients in cluster 2 showed a comparable biomarker profile to the adult AD patients previously stratified into the “Th1/Th2/Th17 dominant” cluster¹⁵, and might thus be considered to have a higher risk for non-resolving AD. Longitudinal follow-up studies are needed to confirm the endotype of each cluster, and to investigate whether the persistence of AD is related to one of the four endotypes and whether endotypes are stable over time or might change after treatment with systemic immunosuppressive or immunomodulatory drugs. Comparing the profile of cleared versus persistent pediatric AD, will better define biomarker-specific characteristics that predict AD clearance.

Despite inclusion of different age groups, this study was not longitudinal and thus did not follow the same cohort over time. Another limitation is the lack of age- and sex-matched healthy controls, which makes it difficult to distinguish disease-specific from age- and sex-specific differences in biomarker profiles, for instance during puberty, although the patient clusters were not influenced by age. The biomarker panel that was measured in this study was based on our previous studies including adult AD patients^{14, 49}, and was not specifically selected for pediatric AD. The panel was composed of all markers available in our laboratory at the moment of measurement. We believe that the broad panel, including many different pathways (Th1/Th2/Th17/Th22, IL-1 family, cell differentiation, skin-homing, innate- and adapted immunity, for example) covers potential adult and pediatric specific markers.

By using an unsupervised profiling approach, the findings of this study indicate that pediatric AD is a biologically heterogeneous disease. We could identify four distinct patient clusters based on serum

biomarker profiles in a large cohort of pediatric AD patients aged 0-17 years. Cluster membership was not influenced by age or age of onset, but disease severity seems to be associated with patient clustering. The identification of endotypes driven by distinct underlying immunopathological pathways might be useful to define pediatric AD patients at risk of persistent disease and may necessitate different targeted treatment approaches. Future longitudinal studies will be needed to further validate the endotypes and may provide additional insights into the stability of endotypes in pediatric AD patients over time.

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TABLES

Table 1. Clinical characteristics of the total cohort of pediatric AD patients

Clinical characteristics	Total group (n=240)
Age (years)†, mean (SD)	8.2 (5.5)
Male, n (%)	100 (41.7)
Female, n (%)	140 (58.3)
EASI score, mean (SD)	14.6 (10.7)
Atopic comorbidities, n (%)	
- allergic asthma	84 (35.0)
- allergic rhinitis	108 (45.0)
- food allergy	87 (36.3)
- no atopic comorbidities	77 (32.1)
Age of onset, n (%)	
- 0 -1 years	180 (75.0)
- 2 – 11 years	48 (20.0)
- 12 – 17 years	2 (0.8)
- missing	10 (4.2)

Categorical variables are presented as counts and percentages; continuous variables are presented as mean with Standard Deviation

(SD). **EASI** Eczema Area Severity Intensity; † age at moment of sample collection.

Table 2. Clinical comparison of the three pediatric AD age groups

Clinical characteristics	0 – 4 years (n=77)	5 – 11 years (n=84)	12 – 17 years (n=79)	p-value
Age (years)[†], mean (SD)	2.0 (1.4)	7.6 (1.9)	14.9 (1.7)	<0.001
Male, n (%)	43 (55.8)	26 (31.0)	31 (39.2)	0.005
Female, n (%)	34 (44.2)	58 (69.0)	48 (60.8)	
EASI score, mean (SD)	13.3 (10.1)	14.3 (11.3)	16.2 (10.5)	0.271
Atopic comorbidities, n (%)				
Allergic asthma	15 (19.5)	30 (35.7)	39 (49.4)	0.001
Allergic rhinitis	15 (19.5)	40 (47.6)	53 (67.1)	<0.001
Food allergy	21 (27.3)	29 (34.5)	37 (46.8)	0.050
No atopic comorbidities	37 (48.1)	27 (32.1)	13 (16.5)	<0.001
Age of onset, n (%)				0.084
0 -1 years	63 (81.8)	64 (76.2)	53 (67.1)	
2 – 11 years	11 (14.3)	16 (19.0)	21 (26.6)	
12 – 17 years	NA	NA	2 (2.5)	
missing	3 (3.9)	4 (4.8)	3 (3.8)	

Categorical variables are presented as counts and percentages; continuous variables are presented as mean with Standard Deviation (SD). Clinical characteristics between the age groups were compared using a one-way ANOVA or chi-square test when appropriate. P values <0.05 were considered statistically significant. **EASI** Eczema Area Severity Intensity; [†] age at moment of sample collection, distribution of ages among age groups are presented in Supplemental Figure E1.

Table 3. Clinical comparison of four serum biomarker-based pediatric AD patient clusters

Clinical characteristics	Cluster 1 (n=98)	Cluster 2 (n=73)	Cluster 3 (n=43)	Cluster 4 (n=25)	p-value
Age (years)[†], mean (SD)	8.7 (5.7)	8.8 (5.3)	6.9 (5.4)	6.6 (4.9)	0.109
min – max	0 – 17	0 – 17	0 – 17	0 – 17	
Male, n (%)	52 (53.1)	23 (31.5)	20 (46.5)	5 (20.0)	0.004
Female, n (%)	46 (46.9)	50 (68.5)	23 (53.5)	20 (40.0)	
EASI score, mean (SD)	9.2 (5.4)	27.8 (7.5)	10.5 (9.1)	12.3 (9.1)	<0.001
Atopic comorbidities, n (%)					
Allergic asthma	39 (39.8)	27 (37.0)	12 (27.9)	6 (24.0)	0.272
Allergic rhinitis	43 (43.9)	38 (52.0)	16 (37.2)	11 (44.0)	0.755
Food allergy	29 (29.6)	39 (53.4)	13 (30.2)	6 (24.0)	0.017
No atopic comorbidities	33 (33.7)	17 (23.3)	16 (37.2)	10 (40.0)	0.267
Age of onset, n (%)					0.384
0 -1 yrs	77 (78.6)	55 (75.3)	26 (60.5)	21 (84.0)	
2 – 11 yrs	17 (17.3)	15 (20.5)	13 (30.2)	3 (12.0)	
12 – 17 yrs	2 (2.0)	0 (0)	0 (0)	0 (0)	
missing	2 (2.0)	3 (4.1)	4 (9.3)	1 (4.0)	

Categorical variables are presented as counts and percentages; continuous variables are presented as mean with Standard Deviation (SD). Clinical characteristics between the patient clusters were compared using a one-way ANOVA or chi-square test when appropriate. P values <0.05 were considered statistically significant. **EASI** Eczema Area Severity Intensity; [†] age at moment of sample collection.

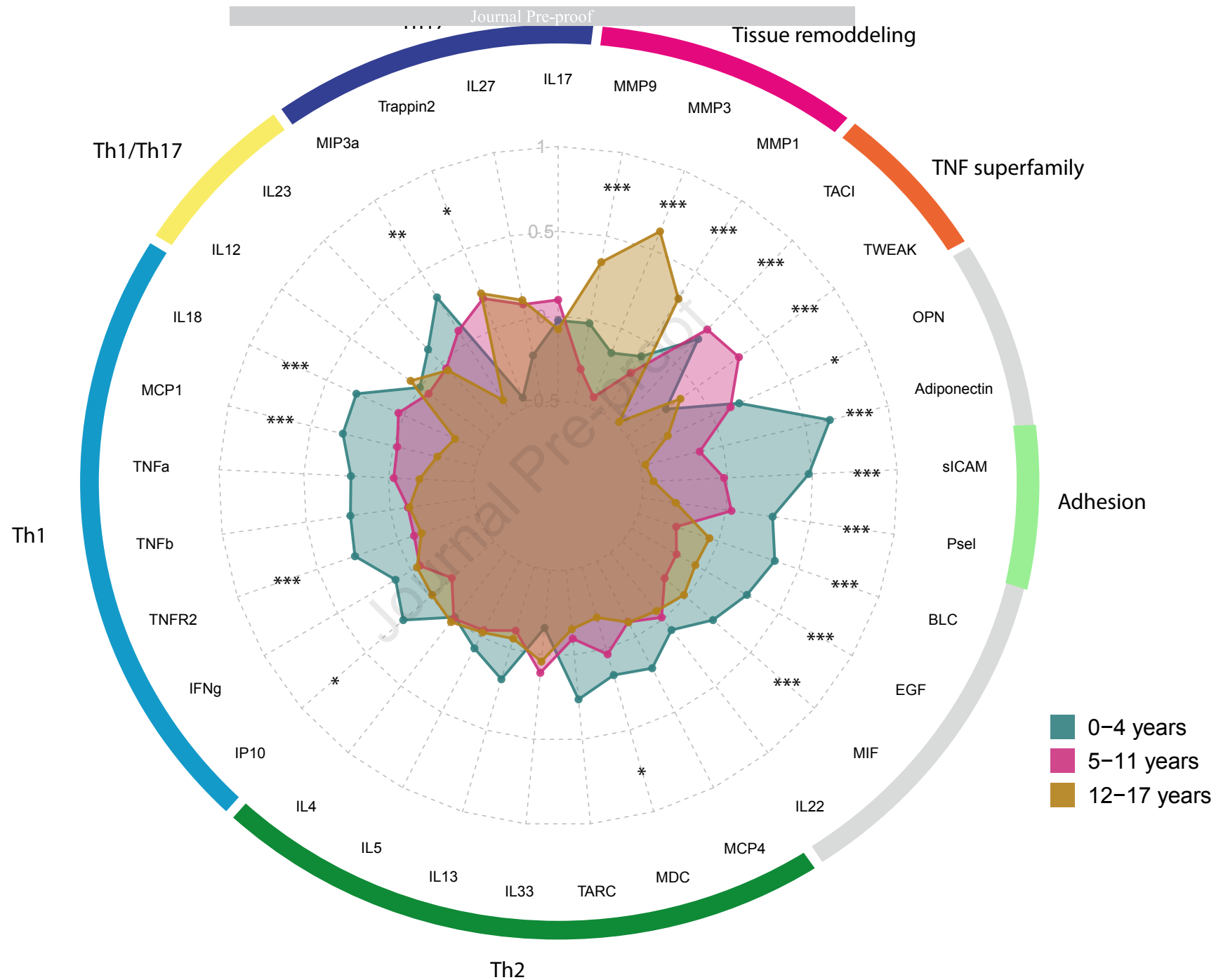
FIGURE LEGENDS

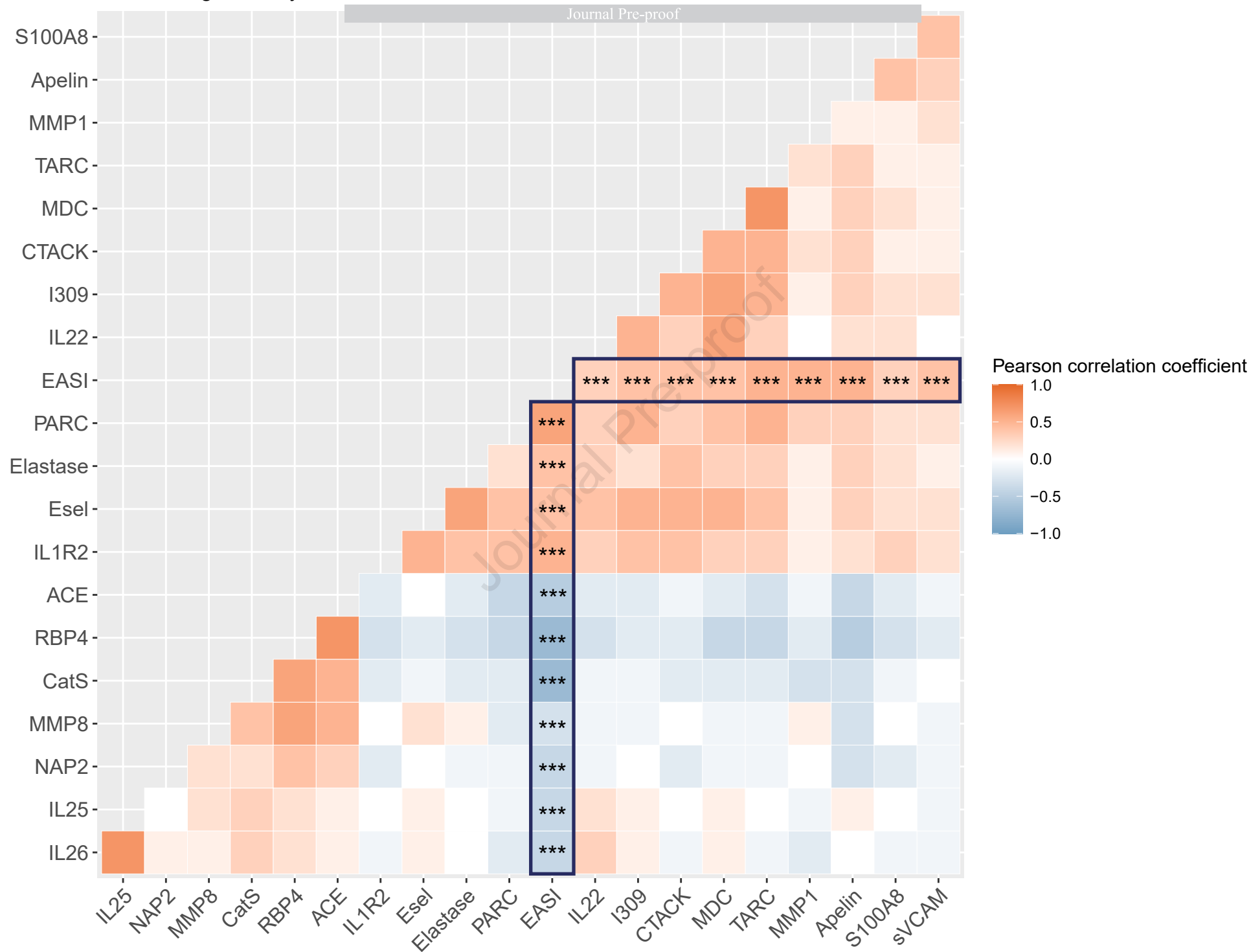
Figure 1. Biomarker profiles in AD children divided in three different age groups. Averages of Box-Cox transformed serum biomarker levels were compared between AD children aged 0 – 4 years, 5 – 11 years, and 12 – 17 years at the moment of sampling. Radar plot shows biomarker profiles per age group for selected markers based on significance and AD-related pathways. Spoke lengths represent means of Box-Cox transformed data per variable. Significance levels for one-way ANOVA results are presented with asterisks. P-values lower than 0.05 were considered statistically significant. Significance levels correspond to the following P-values: *P < 0.05, **P < 0.01, and ***P < 0.001.

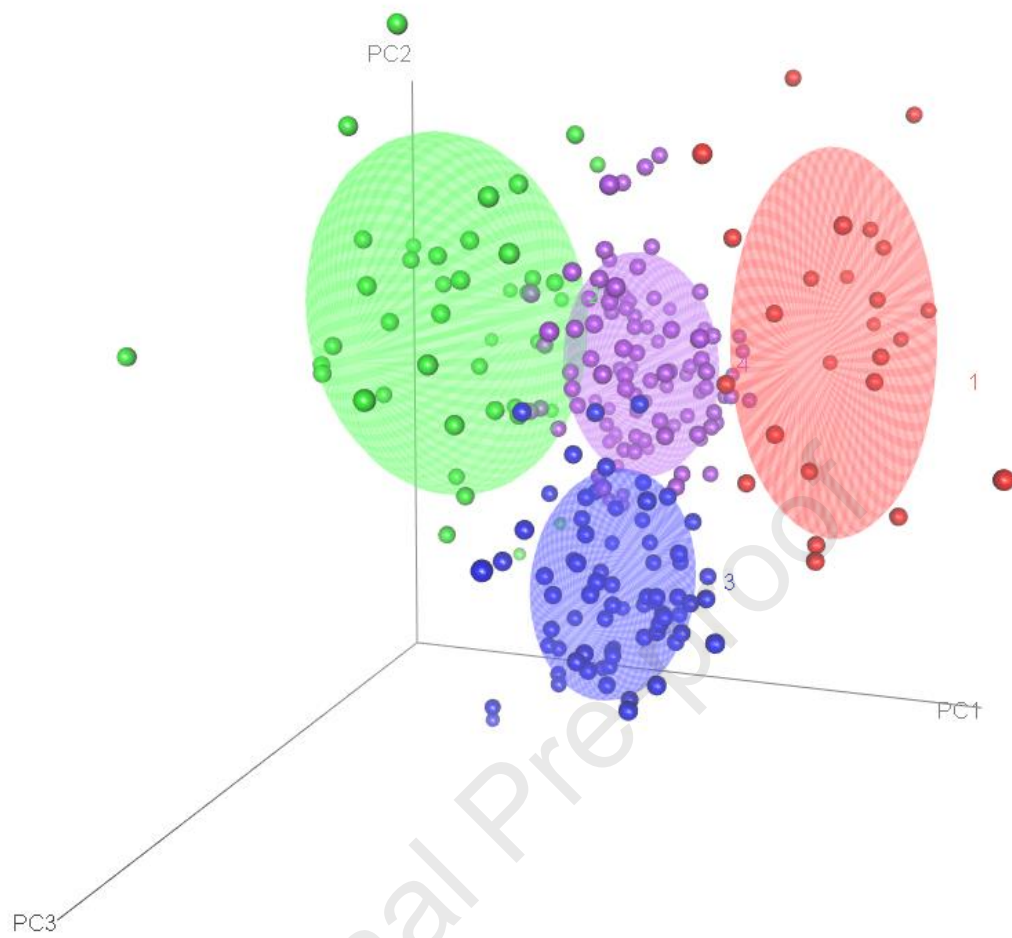
Figure 2. Correlation of disease severity with serum biomarkers in pediatric AD patients. Heatmap of Pearson correlations between serum biomarkers and AD disease severity measured by Eczema Area and Severity Index (EASI). Heatmap includes only serum biomarkers that significantly correlate with EASI score and have Pearson correlation coefficients >0.30 or <-0.30. Red denotes positive and blue denotes negative correlations. Blue boxes mark correlations between serum biomarkers and EASI, *** represents P-value <0.001.

Figure 3. Principal component and cluster analyses of pediatric AD patients. (A) Using unsupervised k-means clustering of the first 50 PCs resulted in the identification of four pediatric AD patient clusters (1, 2, 3, and 4). 239 patients are presented in a three-dimensional plot in terms of the first three PCs. Colors and colored ellipses represent clusters. PC1 explained 23.3% of the variance, PC2 explained 11.7% of the variance and PC3 explained 8.6% of the variance. (B) Two-dimensional plots of 239 patients on the first three PCs. Colors and colored ellipses represent clusters. Symbols represent the three age groups. Patients from all three age groups were equally divided over the four clusters.

Figure 4. Biomarker profiles of four distinct pediatric AD patient clusters. Averages of Box-Cox transformed serum biomarker levels were compared between the four identified pediatric AD clusters (cluster 1, 2, 3, and 4). Radar plot shows biomarker profiles per cluster for markers that were significantly higher or lower expressed in one of the clusters compared to the other clusters. Spoke lengths represent means of Box-Cox transformed data per variable.







PC2 11.7 % explained var.

